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(54) Title: NUCLEIC ACID ENCODING NOVEL EGF-LIKE GROWTH FACTORS

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                    10         20         30         40         50
BOV-AG     1 MKLLPSVVLK LFLAAVLSAL VTGESLERLW RVLAAGASNL DSPTQSTDQL  50
HUM-HB     1 MKLLPSVVLK LFLAAVLSAL VTGESLERLR RGLAAGTSNP DPPTVSTDQL  50
PIG-HB     1 MKLLPSVVLK LFLAAVFSAL VTGESLERLR RGLADGTSNL VSPTESTDQL  50
MUS-HB     1 MKLLPSVMLK LFLAAVLSAL VTGESLERLR RGLAAATSNP DPPTGSTNQL  50
RAT-HB     1 MKLLPSVVLK LFLAAVLSAL VTGESLERLR RGLAAATSNP DPPTGTTNQL  50
                    60         70         80         90        100
BOV-AG    51 LPVGGDQGWE VLDLEEANLD LFRAAFSSKT QALATPSKEE ----------  100
HUM-HB    51 LPLGGGRDRK VRDLQEADLD LLRVTLSSKP QALATPNKEE HGKRKKKGKG  100
PIG-HB    51 LPPGGGRGRE VLDLEEADLD LLRADFSSKP QALATPSKEE RGKRKKKGKG  100
MUS-HB    51 LPTGGDRAQG VQDLEGTDLN LFKVAPSSKP QGLATPSKER NGKKKKKGKG  100
RAT-HB    51 LPTGADRAQE VQDLEGTDLD LFKVAPSSKP QALATPGKEK NGKKKRKGKG  100
                   110        120        130        140        150
BOV-AG   101 -GKKRNPCLR RYKDPCIHGE CKYVKELRVP TCICYPGYYG ERCHGLSLPV  150
HUM-HB   101 LGKKRDPCLR KYKDPCIHGE CKYVKELRAP SCICHPGYHG ERCHGLSLPV  150
PIG-HB   101 LGKKRDPCLR KYKDPCIHGE CKYVKELRAP SCICHPGYHG ERCHGLSLPV  150
MUS-HB   101 LGKKRDPCLR KYKDYCIHGE CRYLQEFRTP SCKCLPGYHG HRCHGLTLPV  150
RAT-HB   101 LGKKRDPCLK KYKDYCIHGE CRYLKELRIP SCHCLPGYHG QRCHGLTLPV  150
                   160        170        180        190        200
BOV-AG   151 KNRLYTY--- ---------- ---------- ---------- ----------  200
HUM-HB   151 ENRLYTYDHT TILAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDVENEEKV  200
PIG-HB   151 KNRLYTYDHT TILAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDVENEEKV  200
MUS-HB   151 ENPLYTYDHT TVLAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDLESEEKV  200
RAT-HB   151 ENPLYTYDHT TVLAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDLESEEKV  200
                   210        220        230        240        250
BOV-AG   201 --------.. .......... .......... .......... ..........  250
HUM-HB   201 KLGMTNSH.. .......... .......... .......... ..........  250
PIG-HB   201 KLGVTASH.. .......... .......... .......... ..........  250
MUS-HB   201 KLGVASSH.. .......... .......... .......... ..........  250
RAT-HB   201 KLGMASSH.. .......... .......... .......... ..........  250
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(57) Abstract: The present invention relates to nucleic acids encoding a protein, which is an epidermal growth factor receptor (EGFR)-ligand having e.g. no heparin binding site. Preferably, the protein is capable of stimulating astroglial cell maturation and/or has a selective survival promoting activity on dopaminergic (DAergic) and/or peripheral neurons and/or has a regenerative effect on peripheral and axonal neurons. The present invention further relates to antisense nucleic acids, ribozymes and antibodies directed to the nucleic acid or the protein, to methods of their production, to antagonists directed to the protein, to agonists which substitute the functional activity of the protein and to pharmaceutical compositions for the treatment as well as to diagnostic kits for the detection of disorders such as neurodegenerative diseases, cancer and AIDS.

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"Nucleic Acid Encoding Novel EGF-Like Growth Factors"

Description

The present invention relates to nucleic acids encoding a protein, which is an epidermal growth factor receptor (EGFR)-ligand having e.g. no heparin-binding site. Preferably, the protein is capable of stimulating astroglial cell maturation and/or has a selective survival promoting activity on dopaminergic (DAergic) and/or peripheral neurons and/or has a regenerative effect on peripheral and axonal neurons. The present invention further relates to antisense nucleic acids, ribozymes and antibodies directed to the nucleic acid or the protein, to methods of their production, to antagonists directed to the protein, to agonists which substitute the functional activity of the protein and to pharmaceutical compositions for the treatment as well as to diagnostic kits for the detection of disorders such as neurodegenerative diseases, cancer and AIDS.

Based on sequence similarity, mitogenic activity, and capability to interact with EGF receptors, there are five distinct members of the epidermal growth factor (EGF) family. These members are EGF, transforming growth factor-alpha (TGF-A), amphiregulin, heparin-binding EGF-like growth factors (HB-EGF), and betacellulin, (Massague and Pandella *Annu. Rev. Biochem.* 62, 515-541; Shing et al. *Science* 259, 1604-1607).

Expression of porcine HB-EGF mRNA derived from 22 different tissues was investigated (Vaughan et al., *Biochem. J.* (1992) 287 681-684). In this study, detection of HB-EGF via RT-PCR techniques shows expression in skin, midbrain, cerebellum, hypothalamus, cerebral cortex, bulbourethral gland, lung, heart (ventricel), kidney, prostate, seminal vesicle and testis. Weak expression was found in lymph node, thymus and spleen, however no HB-EGF expression was found in pituitary, olfactory bulb, thyroid, duodenum, pancreas, liver or submaxillary gland.

Therefore, there is a great demand for novel EGF-like factors that relate to any diseases which can be influenced by binding of said EGF-like factors to the corresponding receptors.

Accordingly, the technical problem underlying the present invention ist to provide novel EGF-like factors and nucleic acids coding for such factors.

The solution to the above technical problem is achieved by the embodiments characterized in the claims.

In particular, the present invention relates to a nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a protein, e.g. derived from chromaffin granules, or a functionally active fragment or derivative or mutant or variant thereof, wherein the protein is an EGFR-ligand, preferably being capable of stimulating astroglial cell maturation and/or having a selective survival promoting activity on DAergic neurons and/or peripheral neurons. In a preferred embodiment, the protein of the present invention has a regenerative effect on peripheral and axonal neurons. The protein of the present invention may have a heparin-binding site or no heparin-binding site.

The term "nucleic acid" and "nucleotide sequence" refer to endogeneously expressed, semi-synthetic, synthetic or chemically modified nucleic acid molecules, preferably consisting substantially of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides. Further, the term "nucleotide sequence" may comprise exons, wherein the nucleotide sequence encodes the primary amino acid sequence of the protein and may be degenerated based on the genetic code. The term "primary amino acid sequence" refers to the sequence of amino acids irrespective of tertiary and quaternary protein structure.

The term "protein derived from chromaffin granules" refers to single, defined proteins or functionally active fragments or derivatives or parts or mutants thereof that, when applied singly or in combinations, exert trophic, survival and differentiation promoting effects on DAergic and peripheral neurons. The expression "selective survival promoting acitivity on DAergic and peripheral

neurons" refers to a proteinaceous activity that may confer, by itself or in combination with other factors present in chromaffin granules, survival and differentiation upon DAergic and peripheral neurons within the nanomolar range or below.

The protein encoded by the nucleotide sequence of the nucleic acid according to the present invention is an epidermal growth factor receptor (EGFR)-ligand. Preferred EGF-receptors are e.g. HER, HER2, HER3 and HER4.

The term "heparin-binding site" refers to a three dimensional arrangement of atoms which is capable of interacting with heparin through any physical and/or chemical interaction. Such interactions comprise covalent binding, electrostatic interactions, hydrogen bonding, Van-der-Waals interactions and hydrophobic interactions. An example of a heparin-binding site is a stretch of eleven amino acids, having a substantial basic character due to seven Lys and/or Arg residues, which is found in HB-EGF molecules. A specific heparin-binding site comprises e.g. amino acids 91 to 101 of human HB-EGF (HUM-HB) as shown in Fig. 3.

The chromaffin granule-derived protein of the present invention is capable of promoting astroglial cell maturation. The expression "capable of astroglial cell maturation" means that within a culture system of embryonic mesencephalic cells or in embryonic and adult mesencephalon, the protein increases the number of astroglial cells visualized by expression of proteins that are specific for this cell type.

Furthermore, the chromaffin granule-derived protein, which is encoded by the nucleotide sequence of the above-defined nucleic acid, is capable of modulating the activity and/or proliferation of non-DAergic cells which include e.g. neuronal cells such as glial progenitor cells as well as non-neuronal cells such as cells derived from adrenal gland, pancreas and other tissues. The expression "capable of modulating the activity and/or proliferation of non-DAergic cells" means that an inhibition or stimulation and/or an increase or decrease in the number of the affected cells is the effect initially caused by the "chromaffin granule-derived protein". In the case of non-DAergic cells such as astroglial cells, this initial effect

is supposed to be the prerequisite for the promotion of survival by a factor secreted by the expanded number of astroglial cells.

Preferably, the nucleic acid according to the present invention is derived from a vertebrate. Preferred vertebrates are mammals such as pigs, cattle, rodents, e.g. mice, rats, rabbits, and primates, e.g. humans. The expression "derived from a vertebrate" means that the gene coding for the protein is transcribed and/or translated in cells of the vertebrate, e.g. the mammal, such that the mRNA and/or the protein is detectable by methods known in the art such as *in situ* hybridization, RT-PCR, Northern or Western blotting.

The functionally acitve form of the above-defined protein or fragment or derivative or part thereof may be a monomeric, dimeric or oligomeric form, or a heteromeric form.

A preferred nucleic acid according to the present invention contains at least the nucleotide sequence shown in Fig. 1A, 2A, 8A or 10A (SEQ ID NO 1, SEQ ID NO 4, SEQ ID NO 6 or SEQ ID NO 8) or a fragment or mutant thereof. Such sequences include allelic derivatives of the nucletide sequence shown in Fig. 1A, 2A, 8A or 10A and nucleotide sequences degenerated as a result of the genetic code for said sequences. They also include nucleotide sequences hybridizing with the nucleotide sequence as defined above. Furthermore, mutant sequences of the above nucleotide sequence may result from the insertion, deletion and/or substitution of one or more nucleotides. An example of a mutant nucleotide sequence of the above-defined nucleic acid is a substitution of the thymidine nucleotide (T) at postion 152 of the nucleotide sequence shown in Fig. 1A (SEQ ID NO 1) by a cytosine nucleotide (C). A further example of a mutant sequence results from a deletion of the guanine nucleotide (G) at position 270 of the nucleotide sequence shown in Fig. 1A (SEQ ID NO 1). The resulting amino acid sequence is shown in Fig. 1C (SEQ ID NO 3). Variants of the above-defined nucleic acid may result from alternative splicing of the primary transcript of the gene coding for the nucleotide sequence of the nucleic acid of the present invention. An alternative splicing may result in an insertion, deletion and/or substitution of one or more nucleotides in the nucleotide sequence of the above-

defined nucleic acid. Other variants of the nucleic acid according to the present invention encode proteins which are derived from the EGFR-ligand by, e.g. post-translational, processing and/or modification.

Although said allelic, degenerate and hybridizing sequences may have structural divergences due to naturally occurring mutations, such as small deletions or substitutions, they will usually still exhibit essentially the same useful properties, allowing their use in basically the same medical or diagnostic applications.
According to the present invention, the term "hybridization" means conventional hybridization conditions, preferably conditions with a salt concentration of 6 x SSC at 62 °C to 66 °C followed by a one-hour wash with 0.6 x SSC, 0.1% SDS at 62 °C to 66 °C.

As described in the below examples, the nucleotide sequences shown in Fig. 1A (cDNA of AG-EGF, SEQ ID NO 1; deduced amino acid sequence see Fig. 1B, SEQ ID NO 2) and Fig. 2A (cDNA of the mature form of HB-EGF, SEQ ID NO 4; deduced amino acid sequence see Fig. 2B, SEQ ID NO 5) were obtained from bovine adrenal gland. The nucleotide sequence shown in Fig. 8A (full-length cDNA of (premature) HB-EGF, SEQ ID NO 6, deduced amino acid sequence see Fig. 8B, SEQ ID NO 7) was cloned from a bovine brain cDNA library whereas the nucleotide sequence shown in Fig. 10A (cDNA of PA-EGF, SEQ ID NO 8, deduced amino acid sequence see Fig. 10B, SEQ ID NO 7) was obtained from bovine pancreas. All proteins, bovine adrenal gland (AG)-EGF, bovine pancreas (PA)-EGF as well as premature and mature bovine HB-EGF, encoded by the cloned nucleotide sequences show a surprisingly strong neurotrophic effect.

In the present invention, cloning was carried out according to the method described below. Once the DNA sequence has been cloned, the preparation of host cells capable of producing e.g. the AG-EGF protein, which has no heparin-binding site, and the production of said protein can be easily accomplished using known recombinant DNA techniques comprising constructing the expression plasmids encoding said protein and transforming a host cell with said expression plasmid, cultivating the transformant in a suitable culture medium, and recovering the product having AG-EGF activity.

In general, diseases which are associated with the expression of the nucleotide sequence of the above-defined nucleic acid containing e.g. the nucleotide sequence encoding AG-EGF, can be treated either by increasing the amount or activity of AG-EGF or by suppressing the amount or activity of AG-EGF. Thus, further embodiments of the present invention relate to an antisense nucleic acid directed to the above-defined nucleic acid and to a ribozyme which is capable of cleaving the above-defined nucleic acid. The inhibition may therefore achieved by masking the mRNA with the antisense nucleic acid or by cleaving the mRNA with the ribozyme.

The production of antisense nucleic acids is well known (see e.g. Weintraub, H. M. 1990, *Scientific American* 262: 40). The antisense nucleic acids hybridize with the respective mRNA and form a double-stranded molecule which can then no longer be translated. The use of antisense nucleic acids is, for example known from Marcus-Sekura, C. J. 1988 (*Anal. Biochem.* 172: 289-295). Ribozymes are RNA molecules which are able to specifically cleave other single-stranded RNA molecules. The production of ribozymes is described for example in Czech, J. 1988, *Amer. Med. Assn.* 260: 3030.

A further embodiment of the present invention relates to a vector containing at least the nucleic acid or the antisense nucleic acid or the ribozyme as defined above. The term "vector" refers to a DNA and/or RNA replicon that can be used for the amplification and/or expression of the nucleotide sequence of the nucleic acid or the antisense nucleic acid or the ribozyme as defined above. The vector may contain any useful control units such as promoters, enhancers, or other stretches of sequence within the 5' and/or 3' regions of the nucleotide sequence serving for the control of its expression. The vector may additionally contain sequences within the 5' and/or 3' region of the nucleotide sequence, that encode amino acid sequences which are useful for the detection and/or isolation of the protein which may be encoded by the nucleotide sequence. Preferably, the vector contains further elements that enable the stable integration of the above-defined nucleic acids into the genome of a host organism and/or the transient expression of the nucleotide sequence of the above-defined nucleic acids. It is also prefered

to use vectors containing selectable marker genes which can be easily selected for transformed cells. The necessary operations are well known to the person skilled in the art.

A further embodiment of the present invention relates to a host organism containing at least the nucleic acid or the antisense nucleic acid or the ribozyme or the vector as defined above. Examples of suitable host organisms include various eucaryotic and procaryotic cells, such as *Bacillus spec.* or *E. coli*, insect cells, plant cells, such as tobacco, potato, or *Arabidopsis*, animal cells such as vertebrate cell lines, e.g. mammalian cell lines such as the Mo, COS or CHO cell line, and fungi such as yeast.

A further embodiment of the present invention relates to the protein itself, which is encoded by the nucleic acid as defined above. Preferred examples of the primary amino acid sequence of the protein according to the present invention include the amino acid sequence shown in Fig. 1B, 1C, 2B, 8B or 10B (SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 5, SEQ ID NO 7 or SEQ ID NO 9) as well as functionally active fragments or derivatives or mutants or variants thereof. A mutation leading to a functionally active mutant of the protein according to the present invention may comprise an insertion, deletion or substitution of one or more amino acids. An example of such a mutant amino acid sequence comprises a substitution of the leucine residue (L) at position 51 of the amino acid sequence shown in Fig. 1B (SEQ ID NO 2) by a proline residue (P). A further example of a mutant amino acid sequence is derived from a deletion of nucleotide 270 in the cDNA encoding AGEGF (cf. Fig. 1A) and results in the amino acid sequence shown in Fig. 1C (SEQ ID NO 3). Functionally acitve variants of the protein according to the present invention include e.g. splice variants such as splice variants of the mature form of the above-defined proteins. Variants of the protein according to the present invention may include an insertion, deletion or substitution of one or more amino acids. Other functionally active variants of the protein according to the present invention include proteins which are derived by, e.g. post-translational, processing and/or modification of the above-defined protein.

A further embodiment of the present invention relates to an antagonist which is

directed to the above-defined protein.

Another embodiment of the present invention relates to an agonist as a substitute for the functional activity of the above-defined protein.

Yet a further embodiment of the present invention relates to an antibody, which may be monoclonal or polyclonal, or a functional fragment thereof directed against the protein or a functional derivative or part thereof as defined above.

A further embodiment of the present invention relates to a method for the production of the nucleic acid, the antisense nucleic acid, the ribozyme, the vector, or the protein as defined above, comprising the steps of:

(a) cultivating the above-defined host organism in a suitable medium under suitable conditions; and

(b) isolating the desired product from the medium and/or the host organisms.

A further embodiment of the present invention relates to a pharmaceutical composition containing the nucleic acids and/or the antisense nucleic acid and/or the ribozyme and/or the vector and/or the protein and/or the antagonist and/or the agonist and/or the antibody as defined above, in a pharmaceutically effective amount, optionally in combination with a pharmaceutically acceptable carrier and/or diluent. The pharmaceutical composition may be used for the prevention and/or treatment of diseases influenced by initiation of protein biosynthesis, especially diseases which interfere with the adrenal gland, kidney (i.e. renal failure), and diseases influenced by changes of cell proliferation and/or differentiation, e.g. different types of cancers such as breast, colorectal, liver, kidney, prostate, ovarian, brain and pancreatic tumors, and neurological diseases such as Parkinson`s disease, Huntington`s chorea, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer`s disease and pathogenesis of traumatic, toxic, inflammatory and metabolic neuropathies, and it may be used in wound healing, preferably corneal wound healing. The pharmaceutical composition according to the present invention can also be therapeutically applied for common diseases such as diabetes, ischemia, trauma, haemotopoietic diseases and rheumatoid arthitis. The pharmaceutical composition according to the present invention may

also be used for the treatment of microbial or viral infections such as AIDS.

Furthermore, the pharmaceutical composition of the present invention can also be used in gene therapy. According to the present invention, a method of treating a patient in order to apply the above-defined pharmaceutical compostion may comprise the transfection of, e.g. the above-defined vector *in vitro* or *in vivo* into patient cells. Alternatively, the method may comprise, as a first step, the transfection of the vector *in vitro* into cells and the subsequent implantation of said transfected cells in a patient.

Furthermore, the application of the pharmaceutical composition is not limited to humans but can include animals, in particular domestic animals, as well.

In another preferred embodiment, the pharmaceutical composition according to the present invention further contains one or more growth factors and/or cytokines such as other EGF-like factors, insuline-like growth factors (e.g. IGF-I, IGF-II), TGF-β-like factors (e.g. TGF, BMP, GDF) and neurofactors (e.g. FGF, NGF, BDNF, neurotrophins).

A further embodiment of the present invention relates to a diagnostic kit containing the nucleic acid and/or the antisense nucleic acid and/or the ribozyme and/or the vector and/or the protein and/or and/or the antagonist and/or the agonist and/or the antibody as defined above. The diagnostic kit according to the present invention may preferably be used for the detection of the disorders as defined above.

Furthermore, the above-defined protein as well as the agonist may be used as an additive for cell culture media (*in vitro*/*in vivo*). Therefore, a further embodiment of the present invention relates to a cell culture medium at least containing the above-defined protein and/or the above-defined agonist. The medium according to the present invention may be used e.g. for the stimulation of cultured cells.

A further embodiment of the present invention relates to a process for the preparation of the above defined chromaffin granule-derived protein, comprising

the steps of isolating chromaffin granules from chromaffin cells and extracting the aqueous-soluble protein content containing the chromaffin granule-derived protein, from the chromaffin granules in a buffer solution. The expression "isolating chromaffin granules from chromaffin cells" comprises subcellular fractionation of chromaffin cell organelles by density gradient centrifugation at e.g. 1.7M sucrose. The expression "extracting the aqueous-soluble protein content containing the chromaffin granule-derived protein" comprises lysis of the organelles obtained as a pellet of the examplified 1.7M sucrose centrifugation in e.g. a 10 mM phosphate buffer at pH 7.0 following twenty minutes freezing at -80°C or below.

Further subject-matter of the present invention relates to the use of a protein or a functionally active fragment or derivative or mutant or variant thereof, wherein the protein is an EGFR-ligand, preferably being capable of stimulating astroglial cell maturation and/or preferably having a selective survival promoting activity on DAergic and/or peripheral neurons, for the preparation of a pharmceutical composition for the prevention and/or treatment of diseases selected from the group consisting of diseases influenced by initiation of protein biosynthesis, diseases influenced by changes of cell proliferation and/or differentiation and neurological diseases. Preferred examples of diseases in the use as defined above may be selected from the diseases as defined above for the pharmaceutical composition of the present invention.

The figures show:

Fig. 1 (A) shows the nucleotide sequence of the cDNA encoding AG-EGF (SEQ ID NO 1) derived from bovine adrenal gland and (B) shows the amino acid sequence of AG-EGF (SEQ ID NO 2) as deduced from the nucleotide sequence shown in (A). (C) shows the amino acid sequence of a mutant AG-EGF (SEQ ID NO 3) resulting from the deletion of nucleotide 270 of the nucleotide sequence shown in (A).

Fig. 2 (A) shows the nucleotide sequence of the cDNA encoding mature

HB-EGF (SEQ ID NO 4) derived from bovine adrenal gland and (B) shows the amino acid sequence of HB-EGF (SEQ ID NO 4) as deduced from the nucleotide sequence shown in (A).

Fig. 3 shows the alignment of the amino acid sequence of AG-EGF with some of the related HB-EGF members. The mature regions are from positions 63 to positions 149. The dashed line from position 91 to 101 in the sequence denoted BOV-AG (SEQ ID NO 2) shows the region of the absent heparin binding-site. BOV-AG: amino acid sequence of AG-EGF (SEQ ID NO 2); HUM-HB: amino acid sequence of human HB-EGF (SEQ ID NO 10, Swiss Prot Accession no. Q99075); PIG-HB: amino acid sequence of porcine HB-EGF (SEQ ID NO 11, Swiss Prot Accession No. Q01580); MUS-HB: amino acid sequence of mouse HB-EGF (SEQ ID NO 12, Swiss Prot Accession No. Q06186); RAT-HB: amino acid sequence of rat HB-EGF (SEQ ID NO 13, Swiss Prot Accession No. Q06175).

Fig. 4 (A) shows an alignment of the amino acid sequences of the mature regions of the following EGF family proteins: BOV-MAT.AMI: amino acid sequence of AG-EGF (SEQ ID NO 2); BHB-MAT.AMI: amino acid sequence of the mature form of bovine HB-EGF (SEQ ID NO 5); HUM-MAT.AMI: amino acid sequence of the mature form of human HB-EGF (SEQ ID NO 10; Swiss Prot Accession No. Q99075); PIG-MAT.AMI: amino acid sequence of the mature form of porcine HB-EGF (SEQ ID NO 11, Swiss Prot Accession No. Q01580); MUS-MAT.AMI: amino acid sequence of the mature form of mouse HB-EGF (SEQ ID NO 12, Swiss Prot Accession No. Q06186); RAT-MAT.AMI: amino acid sequence of the mature form of rat HB-EGF (SEQ ID NO 13, Swiss Prot Accession No. Q06175). (B) shows the plot of a phylogenetic tree of members of the EGF family. The phylogenetic tree was constructed with the computer program DNASIS (Hitachi, France) using the sequences shown in (A) plus the following further members of the EGF family: AMP-MAT.AMI: mature form of human amphiregulin (Swiss Prot

Accession No. P15514); TGF-MAT.AMI: mature form of human TGF-alpha (Swiss Prot Accession No. P01135); BET-MAT.AMI: mature form of human betacellulin (Swiss Prot Accession No. P35070); EGF-MAT.AMI: mature form of human EGF (Swiss Prot Accession No. P001133).

Fig. 5 shows a plot of an elution profile of an affinity-chromatography used for the purification of AG-EGF from chromaffin granules. Left abscissa: absorption at 280 nm (arbitrary units); right abscissa: concentration of buffer B (%); ordinate: time (min) and fraction numbers, respectively.

Fig. 6 shows the image of a Western blot analysis of fractions of the chromatographic run shown in Fig. 5 after SDS-gel electrophoresis (15%) under non-reducing conditions followed by immunostaining using an anti-HB-EGF antibody. Lanes: M: Molecular Weight Marker; 1: crude extract from chromaffin granules; 2: recombinant human HB-EGF; 3: fraction no. 1 from heparinsepharose chromatography; 4: fraction no. 2; 5: fraction no. 3; 6: fraction no. 4; 7:fraction no. 7; 8: fraction no. 8; 9: fraction no. 9; 10: fraction no. 10; 11: fraction no. 11; 12: fraction no. 14; 13: wash; 14: flow through.

Fig. 7 (A) is a diagram demonstrating the survival promoting effect of AG-EGF and HB-EGF from bovine chromaffin granules (VP 1:20). Each bar represents the mean number of TH-positive cells counted in triplicate cultures +/- SEM from two experiments. It is also shown that this neurotrophic effect is inhibited using anti HB-EGF antibody (VP+a-HB-EGF). C: control. The table in (B) shows results of appearance of GFAP positive astroglial cells induced by AG-EGF and HB-EGF (GFAP+ cells) and proliferation of non DAergic cells (PCNA+ cells).

Fig. 8 (A) shows the nucleotide sequence of the full-length cDNA encoding HB-EGF (SEQ ID NO 6) derived from a bovine brain cDNA library.

The coding sequence starts at bp 71. (B) shows the amino acid sequence of HB-EGF (SEQ ID NO 7) as deduced from the coding sequence (nucleotides 71 to 697) shown in (A).

Fig. 9 shows photographic images of agarose gels for the analysis of the distribution of AG/PA-EGF and HB-EGF mRNA in bovine tissues using AG-/HB-EGF specific primers (lanes a) and using β-actin specific primers as positive control (lanes b). M: 100 bp standard ladder; 1: heart; 2: pancreas; 3: kidney; 4: liver; 5: brain; 6: testis; 7: adrenal gland; 8: blood.

Fig. 10 (A) shows the nucleotide sequence of the cDNA encoding PA-EGF (SEQ ID NO 8) derived from bovine pancreas via RT-PCR. (B) shows the amino acid sequence of PA-EGF (SEQ ID NO 9) as deduced from the nucleotide sequence shown in (A).

The following non-limiting examples further illustrate the invention:

EXAMPLES

Cloning of the cDNA encoding bovine AG-EGF and mature bovine HB-EGF

Nucleic acids encoding novel EGF-like growth factors, bovine AG-EGF and bovine HB-EGF, were isolated from bovine adrenal gland.

For the polymerase chain reaction (RT-PCR), poly A^+ RNA, which was isolated from bovine adrenal gland, was used as template in a 50 µl reaction mixture. The PCR reaction was carried out in a RoboCycler Gradient 96 (STRATAGENE, U.S.A.). The amplification was performed with the Titan One Tube RT-PCR System, (Boehringer Mannheim, Germany) as described by the manufacturer, with 30 pmol of each oligonucleotide:

For bovine AG-EGF:

5´- ATGAAGCTGCTGCCGTCGGT -3´ (SEQ ID NO 14).

5´- CTCCT(AG)TG(AG)TA(CT)CT(AG)AACAT -3´(SEQ ID NO 15).

For mature bovine HB-EGF:

5'- CATATGGACTTGGAAGAGGCAAACC -3' (SEQ ID NO 16).

5'- AAGCTTAGAGGCTCAGCCCATGGCACC -3' (SEQ ID NO 17).

The PCR reaction contained 1 µl (1 µg/µl) poly A^+ RNA from bovine adrenal gland. The reaction mixture was incubated for 120 s at 94°C and subjected to 10 cycles (50 s at 94°C, 50 s at 45°C, 50 s at 68°C), followed by 25 cycles (50 s at 94°C, 50 s at 50°C, 50 s at 68°C) with an additional extension for 120 s at 68°C in the thermocycler.

A 10 µl sample from the RT-PCR amplification was fractionated by electrophoresis using a 2 % agarose gel in TBE buffer. After electrophoresis amplified DNA corresponding to a molecular weight of about 600 bp (AG-EGF) and 250 bp (HB-EGF), respectively, was excised from the gel and isolated by using the DNA Gel Extraction Kit (QIAGEN, Germany) following the instructions of the manufacturer.

Cloning of the purified DNA was carried out using the Original TA Cloning Kit (Invitrogen, Germany, Cat. no. K2000-40). Plasmid DNA from positive clones was isolated using the QIAwell 8 Plus Plasmid Kit (QIAGEN, Cat. no. 16142) and sequenced with an automatic DNA sequencer (ALFexpress, PHARMACIA). The resulting DNA sequence was analyzed by a homology search with the Blast program (Genetics Computer Group, Wisconsin Package 9.0, Madison, WI, USA, 1997).

The amino acid sequence alignment of AG-EGF with sequences of different HB-EGF molecules (Figs. 3 and 4A) and the corresponding phylogenetic tree of AG-EGF and other members of the EGF family (Fig. 4B) demonstrate the homology of AG-EGF to the EGF family. The homology of the derived AG-EGF mature amino acid sequence (amino acid 1 to amino acid 75) to the mature form of bovine HB-EGF (amino acid 1 to amino acid 86) displays a sequence homology of 83.7%.

Isolation of AG-EGF and HB-EGF from chromaffin granules

Isolation of soluble chromaffin granule content. The isolation procedures followed essentially the protocol of Winkler and Smith described in *Handbook of Physiology* (Blaschko H, Smith AD, Sayers G, eds), Section 7, Vol. 6, 321-339, 1975. Briefly, approx. 60 bovine adrenals obtained from the slaughter-house, Mannheim, Germany, were dissected, medullae pooled in 0.3 M sucrose, 10 mM phosphate buffer, pH 7.0, homogenized, centrifuged for 15 min at 380 g, followed by centrifugation of the supernatant at 8,720 g for 20 min. The pellet is known to contain all organelles, which can then be further factionated by sucrose gradients. In order to purify large dense core vesicles, the chromaffin granules, the resuspended pellet (0.3 M sucrose, 10 mM phosphate, pH 7.0) was loaded on a 1.7 M sucrose cushion. The chromaffin granules were obtained as a sediment after centrifugation at 100,000 g for 90 min. The sediment was resuspended in 10 mM phosphate pH 7.0, frozen in liquid nitrogen for twenty minutes and subsequently thawed, in order to lyze the vesicles and to extract the soluble protein content. Membrane fragments were collected by centrifugation at 100,000g for 30 min. The supernatent containing the soluble protein mixture from chromaffin granules was dialysed (cutoff 3,500 MW) over night against several batches of 100-fold excess of 10 mM phosphate buffer pH 7.0 to seperate catecholamines and other low molecular weight components from proteins. To quantify protein concentrations the Bradford (Bio-Rad) protein assay was employed using bovine gamma globulin as a standard (Bio-Rad). The protein solution was diluted to give a final protein concentration of 20 mg/ml. This protein solution was then sterile filtered (0.22 µM) and stored in aliquots at -70°C.

Isolation of bovine AG-EGF and HB-EGF by affinity-chromatography using heparin-sepharose. Bovine adrenal gland EGF-like activity (AG-EGF and HB-EGF) was further purified from bovine chromaffin granules using affinity-chromatography. The chromatographic purification was realized using the ÄKTA-Explorer system (Pharmacia Biotech, Germany). For affinity-chromatography, a 5 ml Heparin-Sepharose-cartridge (Econo-Pac, BioRad, Germany) was used.

The first step of the chromatographic procedure comprises a column-equilibration with three column volumes (CV) of loading buffer (10 mM TRIS, pH 7.3, 0.1 %

CHAPS). Then the column was loaded with 125 ml bovine adrenal chromaffin granule pool, using the sample pump with a flow rate of 1.5 ml/min.

Subsequently, unbound sample was washed out with three CV of loading buffer (= buffer A). Elution was carried out stepwise with the elution buffer (= buffer B: 10 mM TRIS, pH 7.3, 0.1 % CHAPS, 2 M NaCl) using the following gradient segments:

Gradient segment 1: 0 to 15 % elution-buffer (buffer B); at 15 % buffer B fraction collecting was started with a fraction size of 5 ml.

Gradient segment 2: elution over 20 min with 15 % buffer B (= 300 mM NaCl); after 20 min increase to 30 % buffer B .

Gradient segment 3: elution over 20 min with 30 % buffer B (= 600 mM NaCl); after 20 min increase to 45 % buffer B.

Gradient segment 4: elution over 20 min with 45 % Puffer B (= 900 mM NaCl); after 20 min increase to 100 % buffer B.

After gradient segment 4 had been finished, the column was cleaned with 2 CV of 100 % buffer B and then reequilibrated with 3 CV of 100 % buffer A.

All steps were carried out at a flow rate of 1,5 ml/min and a wavelength of 280 nm.

A typical profile of the affinity chromatography is shown in Fig. 5: AG-EGF shows an elution pattern using heparin-sepharose chormatography which is different from that of HB-EGF molecules. HB-EGF binds strongly to heparin and is eluted at about 1 M NaCl (Raab, G. et al. (1997) *Biochim. Biophys. Acta* 1333: F179-F199). On the contrary, main fractions containing AG-EGF purified from bovine chromaffin granules eluted between 300 and 600 mM NaCl.

AG-EGF and HB-EGF were detected in the collected fractions by western immunostaining with an anti-human HB-EGF-recognizing polyclonal antibody developed in goat (R&D Systems GmbH Wiesbaden, Catalog-No AF-259-NA, Germany); see Fig. 6.

Neurotrophic effect of AG-EGF and HB-EGF

AG-EGF and HB-EGF purified from bovine chromaffin granules exhibited a strong survival promoting effect on DAergic neurons (Fig. 7A). This neurotrophic effect is inhibited using anti HB-EGF antibody. Furthermore, AG-EGF and HB-EGF exhibited a strong stimulation of astroglial cell maturation as well as a stimulation of proliferation of non-DAergic cells as demonstrated by the appearance of GFAP positive astroglial cells (GFAP+ cells) and the proliferation of PCNA+ cells induced by AG-EGF and HB-EGF (Fig. 7B). The astroglial cell maturation effected by AG-EGF and HB-EGF was inhibited using anti-HB-EGF.

Tissue culture. Mesencephalic cell cultures were essentially established as described by Krieglstein and Unsicker (*Neuroscience* (1994) 63: 1189-1196). In brief, the ventral midbrain floor was dissected from embryonic day (E) 14 Wistar rat fetuses of two litters (20-25 embryos) and collected in CMF. Tissue pieces were enzymatically dissociated using 0.25% trypsin (BioWhittaker) in CMF for 15 min at 37°C. After addition of an equal volume of ice-cold horse serum and 1mg DNAse, cells were triturated with fire-polished and siliconized pasteur pipettes and subsequently washed with DMEM/F12. The single cell suspension (100µl) was seeded on polyornithine (0.1 mg/ml in 15mM borate buffer, pH 8.4, Sigma)-laminin (5µg/ml; Sigma) coated glass cover slips at a density of 200.000 cells/cm^2. Coverslips were incubated in a humified 5% CO_2/95% air atmosphere to allow cells to attach. After two hours coverslips were transferred to 24-well plates (Falcon) containing 750 µl medium. On the following day, and subsequently every three days, 500 µl of the medium was replaced and neurotrophic factors were added at the same time at the given concentrations.

Bovine chromaffin cells were isolated by collagenase perfusion and digestion as previously described and enriched to >95% purity employing Percoll gradient centrifugation (Unsicker et al. (1980) *Neuroscience* 5: 1445-1460; Bieger et al. (1995) *J. Neurochem.* 64: 1521-1527). Chromaffin cells were seeded at 200,000 cells/cm^2 on plastic culture flasks (Falcon; $5x10^6$ cells per $25cm^2$) and maintained in 5 ml of DMEM with N1 supplements for 40h. After washing of cells with prewarmed medium cells were exposed to 2ml DMEM/N1 containing the cholinergic agonist carbachol (10^{-5} M) for 15 min, while control cultures were

treated identically, but without secretagogue (cf. Lachmund et al. (1994) *Neuroscience* 62: 361-370). Conditioned medium from stimulated and unstimulated cells was stored in aliquots at -80°C to avoid repeated freezing and thawing and applied at 1:4 dilution to cultures of mesencephalic DAergic neurons.

Immunocytochemistry. To identify DAergic or serotinergic neurons cells were visualized using antibodies aginst tyrosine hydroxylase (TH), or serotonin, respectively. Cells were fixed with 4% paraformaldehyde buffered in phosphate buffered saline (PBS) for 10 min at room temperature, permeabilized with acetone at -20°C for 10 min and washed with (PBS). After blocking with 1% H_2O_2 in PBS, followed by 1% horse serum, coverslips were stained with a monoclonal antibody to rat TH (1:200; Boehringer Mannheim; diluted in 1% horse serum) or with an antibody against serotonin (1:50; DAKO) for 1 h at 37°C. Specific staining was vizualized using the anti-mouse Vectastain ABC kit in combination with DAB (Camon, Germany). For glial fibrillary acidic protein (GFAP) immunocytochemistry, cells were fixed and permeabilized using aceton at -20°C for 20 min, then washed with PBS and incubated with a monoclonal antibody against GFAP (1:100; Sigma) for 1 h at 37°C. As a secondary antibody TRITC anti-mouse-IgG was used. To monitor cell proliferation bromodesoxyuridine (BrdU) was added to the culture, 24 hours prior fixation, at a final concentration of 10 µM. Cells were washed twice with PBS and fixed with 70% ethanol buffered with 50 mM glycine, pH 2.0, for 20 min at -20°C. Incorporated BrdU was identified using anti-BrdU detection Kit I (Boehringer, Mannheim). BrdU/TH double detection was achieved by first applying the protocol for BrdU followed after another five washes with PBS and by the procedure for TH staining using TRITC anti-mouse-IgG as a secondary antibody. Nuclei were stained with propidium iodide (20 s, 0.1µg/ml). Coverslips were mounted using Aquatex (Merck, Darmstadt).

Evaluation of cell numbers. Survival of DAergic neurons was evaluated by counting all TH-positive neurons in one diagonal strips of the coverslip using 100-fold magnification. This area corresponded to 12% of the total area. Quantification was done in triplicate and in at least two independent experiments. The number of GFAP-positive cells were assessed likewise.

Statistics. The data were analysed by a one-way ANOVA, and the significance of intergroup differences was determined by applying Student's *t*-test. Differences

were considered significant at *P<0.05, **P<0.01, ***P<0.001.

Isolation of a full-length cDNA encoding bovine HB-EGF

Isolation of a full-length cDNA clone of HB-EGF was performed using a commercially available Bovine Brain Lambda cDNA Library (Stratagene, Cat. no. 937719). For screening, a labelled PCR probe was generated from HB-EGF partial cDNA (SEQ ID NO 3; cf. Fig. 2A). The amplification was performed in 1 x PCR-buffer (Qiagen, Germany), 1 mM of dATP, 1 mM of dCTP, 1 mM of dGTP, 0.6 mM of dTTP (Pharmacia, Germany), 0.4 mM of digoxigenin-11-dUTP (Boehringer Mannheim, Germany), 100 pmol of each oligonucleotide bo-AG/HB-mat-N (5'-GACTTGGAAGAGGCAAACCTGG-3'; SEQ ID NO 18) and bo-AG/HB-mat-R (5'-GAGGCTCAGCCCATGGCACC-3'; SEQ ID NO 19) and 1 U of Taq DNA-polymerase (Qiagen, Germany). The PCR mixture was overlaid with 40 µl of paraffin, incubated for 180 s at 94 °C and subjected to 25 cycles (50 s at 94°C, 50 s at 60°C, 50 s at 72°C) with an additional extension for 180 s at 72°C.

Prehybridization of plaque lift filters from cDNA library was performed at 60°C for 4 h in 0.25 M Na_2HPO_4, 7 % SDS, 1 % BSA, 1mM EDTA, pH 7.2. Hybridization was carried out with 50 ng of labelled HB-EGF PCR probe for 15 h under the same buffer conditions as described above for prehybridization. Filters were washed 3 times (5 min, 10 min and 30 min) with 30 mM Na_2HPO_4, 0.1 % SDS at 60°C. Detection of signals was performed using the DIG Luminescent detection kit from Boehringer Mannheim, Germany (Cat. no. 1363514). According to positive signals, 7 clones were isolated and sequenced.

One of the resulting clones represented the full-lenght cDNA-sequence of bovine HB-EGF as shown in Fig. 8A (SEQ ID NO 5). The corresponding amino acid sequence of full-length bovine HB-EGF is shown in Fig. 8B (SEQ ID NO 6).

Expression of AG-/HB-EGF mRNA in bovine tissues

Relative expression of the AG/HB-EGF gene was determined by RT-PCR analyses. Bulls were killed at a local slaughterhouse. Tissue samples were removed, snap-frozen in liquid nitrogen and stored at –70 °C until needed. Total RNA isolation was performed using the Rneasy Kit (Qiagen) following the instructions by the manufacturer. RT-PCRs were performed with 1 µg of total RNA using the Ready-To-Go RT-PCR Beads (Pharmacia). The reactions were set up as described by the manufacturer, using the Two-Step Protocol: First strand cDNA synthesis was carried out using an oligo $d(T)_{12-18}$ primer at 42 °C for 30 min, PCR amplification was performed using the above primers bo-AG/HB-mat-N and bo-AG/HB-mat-R. The PCR mixture was incubated for 60 s at 95°C and subjected to 35 cycles (60 s at 95°C, 60 s at 55°C, 60 s at 72°C) with an additional extension for 300 s at 72°C. PCR products were analyzed by agarose gel electrophoresis.

Eight different bovine tissues samples (heart, pancreas, kidney, liver, brain, testis, adrenal gland and blood) were examined for AG-/HB-EGF gene expression. Additionally, for each tissue a 460 bp fragment of the ß-actin gene was amplified as a positive control for RT-PCR.

Expression of bovine HB-EGF mRNA was found in all tissues which were investigated (Fig. 9). Main expression of PA/AG-EGF was detected in pancreas, liver, brain and adrenal gland. Low expression of PA/AG-EGF was detected in heart, testis and kidney. No expression of PA/AG-EGF was found in the blood sample, but this sample showed an additional amplification product which is larger than the HB-EGF PCR product.

Cloning and sequencing of the PCR product (lower band) from pancreas (PA-EGF; cf. Fig. 10A; SEQ ID NO 8) revealed a partially different DNA-Sequence when compared with the sequence of AG-EGF shown in Fig.1A (SEQ ID NO 1). These sequence differences result in a partially different reading frame. The corresponding amino acid sequence of PA-EGF is shown in Fig. 10B (SEQ ID NO 9).

Expression of the mature forms of AG-EGF and HB-EGF in E. coli

The mature forms (SphI/PstI fragments) of bovine AG-EGF, bovine HB-EGF and human HB-EGF were each subcloned into the corresponding sites of the expression plasmid pQE-31 (Qiagen, Germany). This cloning strategy resulted in a tag of 6 additional histidine residues at the N-terminus of the mature AG-/HB-EGF molecules. Resulting plasmids were transformed into *E.coli* strain SG13009rep4 from Qiagen (Germany). For expression of the corresponding mature protein, 500 ml of LB (amp, kan) were inoculated with a 5 ml overnight culture, and grown for 180 min at 37°C and 120 rpm until the OD_{600} reached between 0.7 and 0.8. After induction with IPTG (1mM final concentration), the cultures were further incubated for 4 h under the same growth conditions.

The cells were harvested by centrifugation at 6000 rpm for 20 min and the pellets were frozen at –20 °C until further purification.

Purification of recombinant AG-/HB-EGF using Ni-NTA columns

Recombinantly expressed His-tagged AG-/HB-EGF proteins were purified using metalchelate chromatography (Ni-NTA). Frozen *E. coli* material which contained the recombinant protein was resuspended in 20 ml 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0 and agitated on a horizontal shaker for 1 h at room temperature (RT). The suspension was subjected to centrifugation at 10000 x g for 20 min and the supernatant incubated with 2 ml of Ni-NTA-agarose (Qiagen) for 1 h with slow shaking. The whole material was transferred into a suitable column and flowthroughs were collected for further analysis. Elution of the 6xHis-proteins was carried out by 8 M urea and decreasing pH-step gradients of pH 6.3 (wash step, 3 times) and elution at pH 5.9 and pH 4.5. All fractions were collected and analyzed on western blots for the presence of 6xHis-proteins using a commercially available RGS-6xHis-antibody (Qiagen). The main amount of protein eluted at pH 4.5.

Renaturation of recombinant AG-/HB-EGF

Solubilization and refolding of recombinant fusion proteins was performed under a variety of experimental conditions. Method I, a single step method used to renature AG-/HB-EGF molecules, utilizes high concentrations of urea. This method shows significant refolding amounts after a short incubation time of 1 hour on ice using 5 mg protein in 8 M urea, pH 4.5 (total volume: 0.5 ml). Refolding efficiency was increased considerably by incubation at 4 °C for a longer time. For example, an incubation period of more than 4 months was examined showing no loss of biological activity. After refolding, protein was desalted using Microcon 10 columns (Amicon) and washed twice with PBS. Method II, a redox system, utilizes glutathione/glutathione disulfide (GSH/GSSG) in order to renature recombinant AG-/HB-EGF. The refolding mixture is composed of 1 ng/µl protein in 1.2 M urea, 120 mM NaCl, 20 mM Tris/HCl pH 7.5, 5 mM EDTA and glutathione, glutathione disulfide in concentrations of 6 mM and 1 mM, respectively. The mixture was incubated at 4 °C for 48 hours.

Immunological detection of His-tagged AG-/HB-EGF

Immunological detection of 6xHis-tagged AG-/HB-EGF molecules was performed by western blotting using a commercially available RGS-His antibody (Qiagen) against the His-tag in combination with Western Light chemoluminescent detection system using the goat anti-mouse-AP antibody (Tropix, U.S.A.).

Activity assay for recombinant AG-/HB-EGF employing EP170.7 cells

EP170.7 cells undergo apoptosis in the absence of interleukin-3 (IL-3), but as they express the EGF-receptor, they can survive in the presence of EGF receptor ligand by using a different pathway.

Cells were grown in IL3-supplemented RPMI 1640 medium (Gibco) with 10% FCS at 37 °C and 5 % CO_2 to a density of 1.5 x 10^6 /ml and washed once in IL3-free medium. 0.5 ml of cell suspension were added to 2.5 ml IL3-free medium in 3 cm dishes (6-well plate) and the proteins of interest were given directly to the wells.

After incubation for two days, numbers of cells were counted with the Neubauer-chamber. The living and dead cells were counted and set in relation to the total cell number. The ratio is a measure of the activity of the tested protein.

Results of the activity assay of bovine AG-EGF, bovine HB-EGF and human HB-EGF are shown in Table 1. With respect to bovine HB-EGF and AG-EGF, respectively, proteins which had been subjected to the above-described refolding methods I or II, respectively, were applied in the apoptosis assay. Human HB-EGF was obtained from R&D systems (USA, Cat. No. 259-HE). IL-3 was applied in the form of conditioned media produced by WEHI cells. GDF-5 was manufactured by the applicant.

Table 1

Protein	Assay 1	Assay 2	Assay 3	Assay 4
	[% surviving cells]			
with interleukin-3 (+ IL-3)	87	85	91	85
without interleukin-3 (+ IL-3)	0	0	0	0
GDF-5 (100 ng)	0	0	0	0
human HB-EGF (100 ng)	82	85	73	85
	Refolding Method I		**Refolding Method II**	
	[% surviving cells]			
bovine HB-EGF (100 ng)	78	83	74	83
bovine AG-EGF (100 ng)	80	85	24	34

The results demonstrate a substantial increase in the number of surviving cells for both bovine HB- as well as AG-EGF. Furthermore, the above apoptosis assay shows that refolding method I as well as refolding method II leads to functionally active proteins.

"Nucleic Acid Encoding Novel EGF-Like Growth Factors"

Claims

1. A nucleic acid containing a nucleotide sequence encoding the primary amino acid sequence of a protein or a functionally active fragment or derivative or mutant or variant thereof, wherein the protein is an EGFR-ligand having no heparin-binding site.

2. The nucleic acid of claim 1, wherein the protein is capable of stimulating astroglial cell maturation and/or has a selective survival promoting activity on DAergic and/or peripheral neurons.

3. The nucleic acid according to claim 1 or 2, wherein the protein is derived from chromaffin granules.

4. The nucleic acid according to anyone of claims 1 to 3, wherein the protein is capable of modulating the activity and/or proliferation of non-DAergic cells.

5. The nucleic acid according to anyone of claims 1 to 4, which is derived from a vertebrate.

6. The nucleic acid according to anyone of claims 1 to 5, which contains at least the nucleotide sequence shown in Fig. 1A (SEQ ID NO 1) or Fig. 10A (SEQ ID NO 8) or a fragment or mutant or variant thereof.

7. An antisense nucleic acid directed against the nucleic acid according to anyone of claims 1 to 6.

8. A ribozyme capable of cleaving the nucleic acid according to anyone of claims 1 to 6.

9. A vector containing at least the nucleic acid according to anyone of claims 1 to 6 or the antisense nucleic acid of claim 7 or the ribozyme of claim 8.

10. A host organism containing at least the nucleic acid according to anyone of claims 1 to 6 or the antisense nucleic acid of claim 7 or the ribozyme of claim 8 or the vector of claim 9.

11. A protein encoded by the nucleic acid according to anyone of claims 1 to 6.

12. The protein of claim 11 at least containing the amino acid sequence shown in Fig. 1B (SEQ ID NO 2) or Fig. 1C (SEQ ID NO 3) or Fig. 10B (SEQ ID NO 9) or a functionally active fragment or derivative or mutant or variant thereof.

13. An antagonist directed to the protein of claim 11 or 12.

14. An agonist which substitutes the functional activity of the protein of claim 11 or 12.

15. An antibody or a functional fragment thereof directed against the protein of claim 11 or 12.

16. A method for the production of the nucleic acid according to anyone of claims 1 to 6 or the antisense nucleic acid of claim 7 or the ribozyme of claim 8 or the vector of claim 9 or the protein of claim 11 or 12, comprising the steps of:

(a) cultivating the host organism of claim 10 in a suitable medium under suitable conditions; and

(b) isolating the desired product from the medium and/or the host organisms.

17. A pharmaceutical composition containing the nucleic acid according to anyone of claims 1 to 6 and/or the antisense nucleic acid of claim 7 and/or the ribozyme of claim 8 and/or the vector of claim 9 and/or the protein of claim 11 or 12 and/or the antagonist of claim 13 and/or the agonist of claim 14 and/or the antibody of claim 15, in a pharmaceutically effective amount, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

18. The pharmaceutical composition of claim 17 for the treatment of ischemia, trauma, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, diabetes, haematopoietic diseases, renal failure, rheumatoid arthitis, AIDS, cancer, wound healing and pathogenesis of traumatic, toxic, inflammatory and metabolic neuropathies.

19. The pharmaceutical composition of claim 17 or 18, further containing one or more growth factors and/or cytokines.

20. The pharmaceutical composition of claim 19, wherein the growth factor or cytokine is selected from EGF-like fators, insuline-like growth factors, TGF-β-like factors and neurofactors.

21. A method for treating a patient comprising the step of transfecting the nucleic acid according to anyone of claims 1 to 6 and/or the antisense nucleic acid of claim 7 and/or the ribozyme of claim 8 and/or the vector of claim 9 *in vitro* or *in vivo* into cells.

22. A diagnostic kit containing the nucleic acid according to anyone of claims 1 to 6 and/or the antisense nucleic acid of claim 7 and/or the ribozyme of claim 8 and/or the vector of claim 9 and/or the protein of claim 11 or 12 and/or the antagonist of claim 13 and/or the agonist of claim 14 and/or the antibody of claim 15.

23. The kit according to claim 22 for the detection of ischemia, trauma, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, diabetes, haematopoietic diseases, renal failure, rheumatoid arthitis, AIDS, cancer, wound healing and pathogenesis of traumatic, toxic, inflammatory and metabolic neuropathies.

24. A cell culture medium at least containing the protein according to claim 11 or 12 and/or the agonist according to claim 14.

25. The medium according to claim 24 for the stimulation of cultured cells.

26. A method for the preparation of the protein of claim 11 or 12, comprising the steps of isolating chromaffin granules from chromaffin cells and extracting the aqueous-soluble protein content containing the chromaffin granule-derived protein, from the chromaffin granules in a buffer solution.

27. Use of a protein or a functionally active fragment or derivative or mutant or variant thereof, wherein the protein is an EGFR-ligand, for the preparation of a pharmceutical composition for the prevention and/or treatment of

diseases selected from the group consisting of diseases influenced by initiation of protein biosynthesis, diseases influenced by changes of cell proliferation and/or differentiation and neurological diseases.

28. Use of claim 27, wherein the disease is selected from ischemia, trauma, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, diabetes, haematopoietic diseases, renal failure, rheumatoid arthitis, AIDS, cancer, wound healing and pathogenesis of traumatic, toxic, inflammatory and metabolic neuropathies.

Fig. 1A

```
ATGAAGCTGC TGCCGTCGGT GGTGCTGAAG CTCTTTCTTG CTGCAGTGCT TTCGGCGTTG
GTGACTGGCG AGAGCCTGGA GCGGCTTTGG AGAGTCCTGG CTGCTGGAGC CAGCAATCTG
GACTCCCCTA CTCAATCTAC GGACCAGCTG CTGCCCGTGG GAGGTGACCA AGGCTGGGAA
GTCCTGGACT TGGAAGAGGC AAACCTGGAC CTTTTCAGAG CTGCTTTCTC CTCCAAGACA
CAAGCTCTGG CCACACCCAG CAAGGAGGAG GGGAAGAAGA GAAACCCATG TCTTCGGAGA
TACAAGGACT TCTGCATCCA CGGAGAATGC AAATACGTGA AGGAGCTCCG GGTTCCAACC
TGCATCTGCT ACCCAGGTTA TTATGGAGAG AGGTGCCATG GGCTGAGCCT CCCAGTGAAA
AATCGCTTAT ATACATAC
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Fig. 1B

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MKLLPSVVLK LFLAAVLSAL VTGESLERLW RVLAAGASNL DSPTQSTDQL LPVGGDQGWE
VLDLEEANLD LFRAAFSSKT QALATPSKEE GKKRNPCLRR YKDFCIHGEC KYVKELRVPT
CICYPGYYGE RCHGLSLPVK NRLYTY
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Fig. 1C

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  1  MKLLPSVVLK LFLAAVLSAL VTGESLERLW RVLAAGASNL DSPTQSTDQL

 51  LPVGGDQGWE VLDLEEANLD LFRAAFSSKT QALATPSKEE GRRETHVFGD

101  TRTSASTENA NT
```

Fig. 2A

```
        10         20         30         40         50         60
GACTTGGAAG AGGCAAACCT GGACCTTTTC AGAGCTGCTT TCTCCTCCAA GCCACAAGCT
        70         80         90        100        110        120
CTGGCCACAC CAAGCAAGGA GGAGCGTGGG AAAAGAAAGA AGAAAGGCAA GGGGTTAGGG
       130        140        150        160        170        180
AAGAAGAGAA ACCCATGTCT TCGGAGATAC AAGGACTTCT GCATCCACGG AGAATGCAAA
       190        200        210        220        230        240
TACGTGAAGG AGCTCCGGGT TCCAACCTGC ATCTGCCACC CAGGTTATCA CGGAGAGAGG
       250        260        270        280        290        300
TGCCATGGGC TGAGCCTC
```

Fig. 2B

```
        10         20         30         40         50         60
DLEEANLDLF RAAFSSKPQA LATPSKEERG KRKKKGKGLG KKRNPCLRRY KDFCIHGECK
        70         80         90        100        110        120
YVKELRVPTC ICHPGYHGER CHGLSL
```

Fig. 3

```
                    10         20         30         40         50
BOV-AG       1 MKLLPSVVLK LFLAAVLSAL VTGESLERLW RVLAAGASNL DSPTQSTDQL  50
HUM-HB       1 MKLLPSVVLK LFLAAVLSAL VTGESLERLR RGLAAGTSNP DPPTVSTDQL  50
PIG-HB       1 MKLLPSVVLK LFLAAVFSAL VTGESLERLR RGLADGTSNL VSPTESTDQL  50
MUS-HB       1 MKLLPSVMLK LFLAAVLSAL VTGESLERLR RGLAAATSNP DPPTGSTNQL  50
RAT-HB       1 MKLLPSVVLK LFLAAVLSAL VTGESLERLR RGLAAATSNP DPPTGTTNQL  50
                    60         70         80         90        100
BOV-AG      51 LPVGGDQGWE VLDLEEANLD LFRAAFSSKT QALATPSKEE ---------- 100
HUM-HB      51 LPLGGGRDRK VRDLQEADLD LLRVTLSSKP QALATPNKEE HGKRKKKGKG 100
PIG-HB      51 LPPGGGRGRE VLDLEEADLD LLRADFSSKP QALATPSKEE RGKRKKKGKG 100
MUS-HB      51 LPTGGDRAQG VQDLEGTDLN LFKVAFSSKP QGLATPSKER NGKKKKKGKG 100
RAT-HB      51 LPTGADRAQE VQDLEGTDLD LFKVAFSSKP QALATPGKEK NGKKKRKGKG 100
                   110        120        130        140        150
BOV-AG     101 -GKKRNPCLR RYKDFCIHGE CKYVKELRVP TCICYPGYYG ERCHGLSLPV 150
HUM-HB     101 LGKKRDPCLR KYKDFCIHGE CKYVKELRAP SCICHPGYHG ERCHGLSLPV 150
PIG-HB     101 LGKKRDPCLR KYKDFCIHGE CKYVKELRAP SCICHPGYHG ERCHGLSLPV 150
MUS-HB     101 LGKKRDPCLR KYKDYCIHGE CRYLQEFRTP SCKCLPGYHG HRCHGLTLPV 150
RAT-HB     101 LGKKRDPCLK KYKDYCIHGE CRYLKELRIP SCHCLPGYHG QRCHGLTLPV 150
                   160        170        180        190        200
BOV-AG     151 KNRLYTY--- ---------- ---------- ---------- ---------- 200
HUM-HB     151 ENRLYTYDHT TILAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDVENEEKV 200
PIG-HB     151 KNRLYTYDHT TILAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDVENEEKV 200
MUS-HB     151 ENPLYTYDHT TVLAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDLESEEKV 200
RAT-HB     151 ENPLYTYDHT TVLAVVAVVL SSVCLLVIVG LLMFRYHRRG GYDLESEEKV 200
                   210        220        230        240        250
BOV-AG     201 --------.. .......... .......... .......... .......... 250
HUM-HB     201 KLGMTNSH.. .......... .......... .......... .......... 250
PIG-HB     201 KLGVTASH.. .......... .......... .......... .......... 250
MUS-HB     201 KLGVASSH.. .......... .......... .......... .......... 250
RAT-HB     201 KLGMASSH.. .......... .......... .......... .......... 250
```

Fig. 4A

```
                                  10          20          30          40          50
BOV-MAT.AMI             1 DLEEANLDLF RAAFSSKTQA LATPSKEE-- ---------G KKRNPCLRRY
50
BHB-MAT.AMI             1 DLEEANLDLF RAAFSSKPQA LATPSKEERG KRKKKGKGLG KKRNPCLRRY
50
HUM-MAT.AMI             1 DLQEADLDLL RVTLSSKPQA LATPNKEEHG KRKKKGKGLG KKRDPCLRKY
50
PIG-MAT.AMI             1 DLEEADLDLL RADFSSKPQA LATPSKEERG KRKKKGKGLG KKRDPCLRKY
50
MUS-MAT.AMI             1 DLEGTDLNLF KVAFSSKPQG LATPSKERNG KKKKKGKGLG KKRDPCLRKY
50
RAT-MAT.AMI             1 DLEGTDLDLF KVAFSSKPQA LATPGKEKNG KKKRKGKGLG KKRDPCLKKY
50
                                  60          70          80
BOV-MAT.AMI            51 KDFCIHGECK YVKELRVPTC ICYPGYYGER CHGLSL
100
BHB-MAT.AMI            51 KDFCIHGECK YVKELRVPTC ICHPGYHGER CHGLSL
100
HUM-MAT.AMI            51 KDFCIHGECK YVKELRAPSC ICHPGYHGER CHGLSL
100
PIG-MAT.AMI            51 KDFCIHGECK YVKELRAPSC ICHPGYHGER CHGLSL
100
MUS-MAT.AMI            51 KDYCIHGECR YLQEFRTPSC KCLPGYHGHR CHGLTL
100
RAT-MAT.AMI            51 KDYCIHGECR YLKELRIPSC HCLPGYHGQR CHGLTL
100
```

Fig. 4B

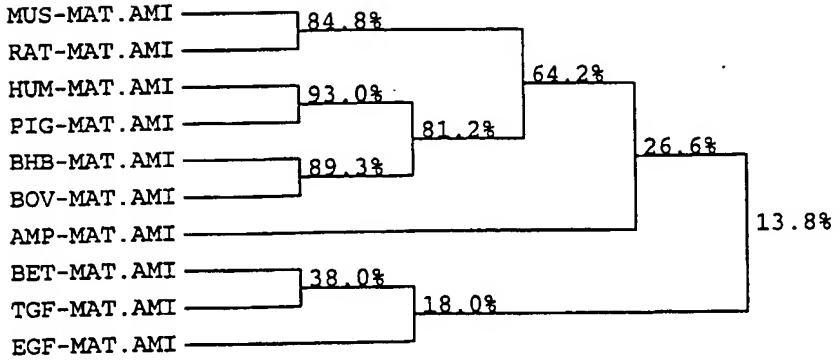


Fig. 5

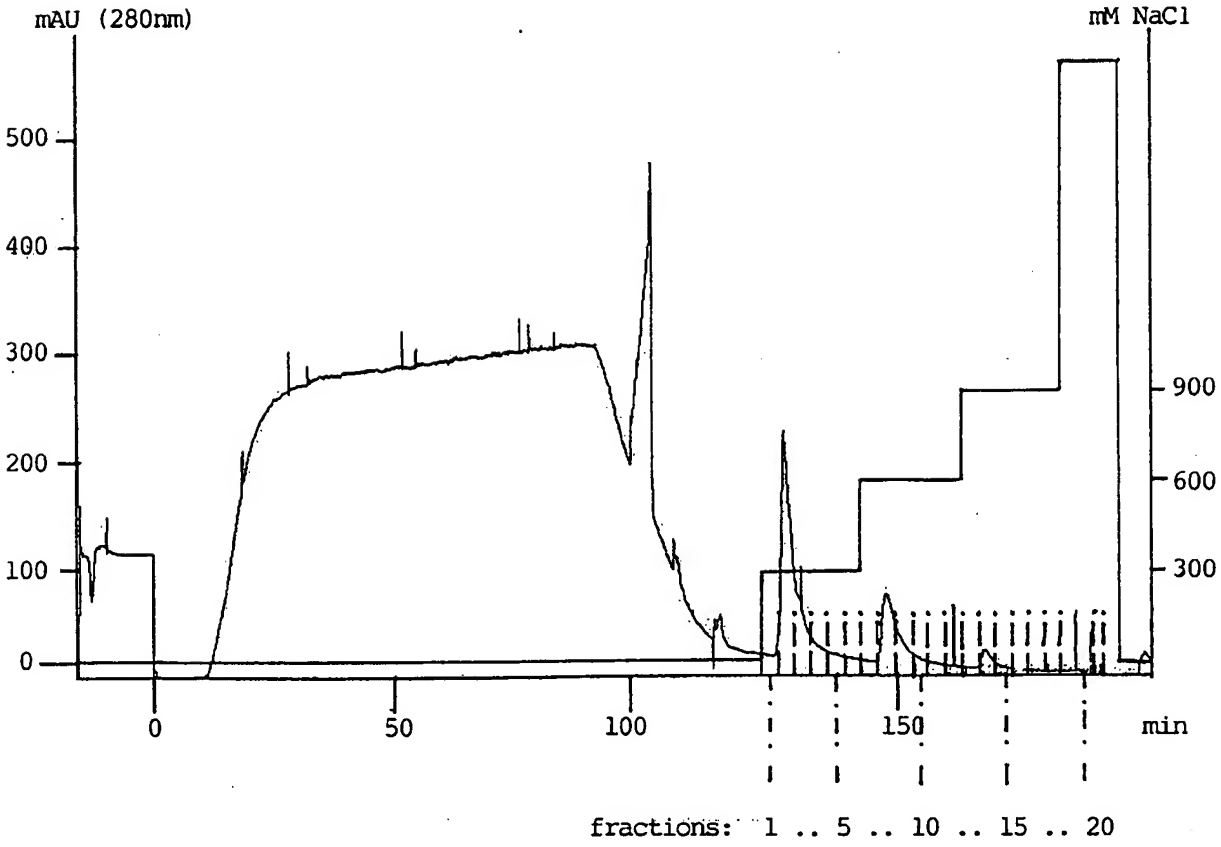

mAU (280nm)
mM NaCl
500
400
300
200
100
0
900
600
300
0
50
100
150
min
fractions: 1 .. 5 .. 10 .. 15 .. 20

Fig. 6

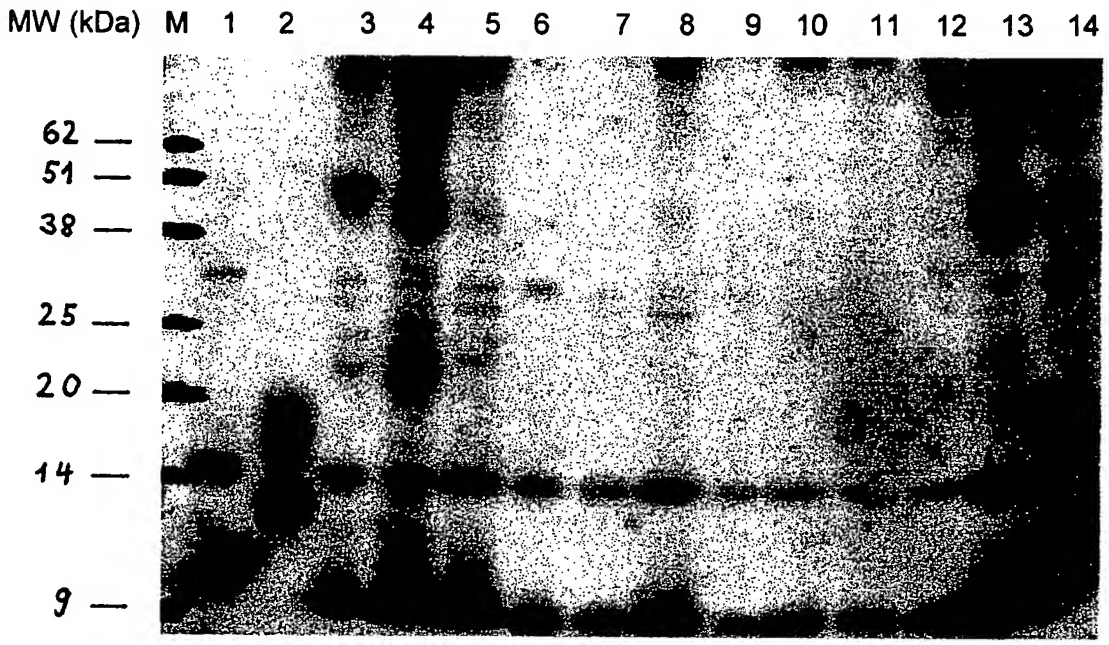


Fig. 7A

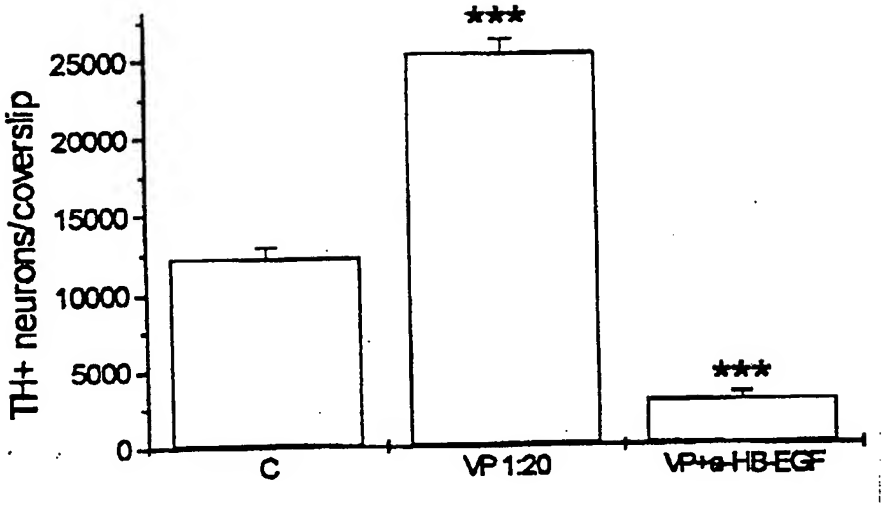


Fig. 7B

	GFAP+ cells:	PCNA+ cells
Control	-/+	++
VP1:20	++++	+++
VP 1:20 +a-HB-EGF (10µg/ml)	-	++

Fig. 8A

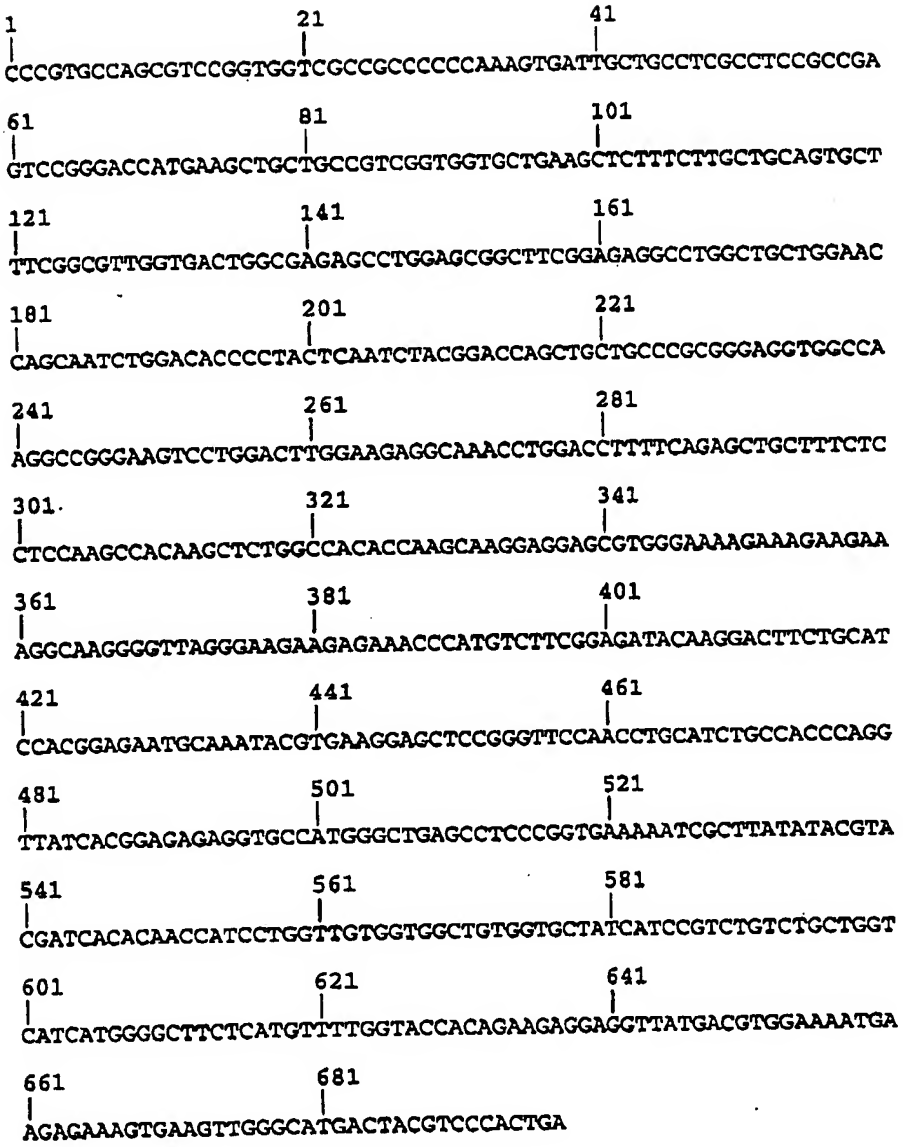

1
21
41
CCCGTGCCAGCGTCCGGTGGTCGCCGCCCCCCAAAGTGATTGCTGCCTCGCCTCCGCCGA
61
81
101
GTCCGGGACCATGAAGCTGCTGCCGTCGGTGGTGCTGAAGCTCTTTCTTGCTGCAGTGCT
121
141
161
TTCGGCGTTGGTGACTGGCGAGAGCCTGGAGCGGCTTCGGAGAGGCCTGGCTGCTGGAAC
181
201
221
CAGCAATCTGGACACCCCTACTCAATCTACGGACCAGCTGCTGCCCGCGGGAGGTGGCCA
241
261
281
AGGCCGGGAAGTCCTGGACTTGGAAGAGGCAAACCTGGACCTTTTCAGAGCTGCTTTCTC
301
321
341
CTCCAAGCCACAAGCTCTGGCCACACCAAGCAAGGAGGAGCGTGGGAAAAGAAAGAAGAA
361
381
401
AGGCAAGGGGTTAGGGAAGAAGAGAAACCCATGTCTTCGGAGATACAAGGACTTCTGCAT
421
441
461
CCACGGAGAATGCAAATACGTGAAGGAGCTCCGGGTTCCAACCTGCATCTGCCACCCAGG
481
501
521
TTATCACGGAGAGAGGTGCCATGGGCTGAGCCTCCCGGTGAAAAATCGCTTATATACGTA
541
561
581
CGATCACACAACCATCCTGGTTGTGGTGGCTGTGGTGCTATCATCCGTCTGTCTGCTGGT
601
621
641
CATCATGGGGCTTCTCATGTTTTGGTACCACAGAAGAGGAGGTTATGACGTGGAAAATGA
661
681
AGAGAAAGTGAAGTTGGGCATGACTACGTCCCACTGA

Fig. 8B

```
          81                  101                 121
M K L L P S V V L K L F L A A V L S A L

         141                 161                 181
V T G E S L E R L R R G L A A G T S N L

         201                 221                 241
D T P T Q S T D Q L L P A G G G Q G R E

         261                 281                 301
V L D L E E A N L D L F R A A F S S K P

         321                 341                 361
Q A L A T P S K E E R G K R K K K G K G

         381                 401                 421
L G K K R N P C L R R Y K D F C I H G E

         441                 461                 481
C K Y V K E L R V P T C I C H P G Y H G

         501                 521                 541
E R C H G L S L P V K N R L Y T Y D H T

         561                 581                 601
T I L V V V A V V L S S V C L L V I M G

         621                 641                 661
L L M F W Y H R R G G Y D V E N E E K V

         681
K L G M T T S H -
```

Fig. 9

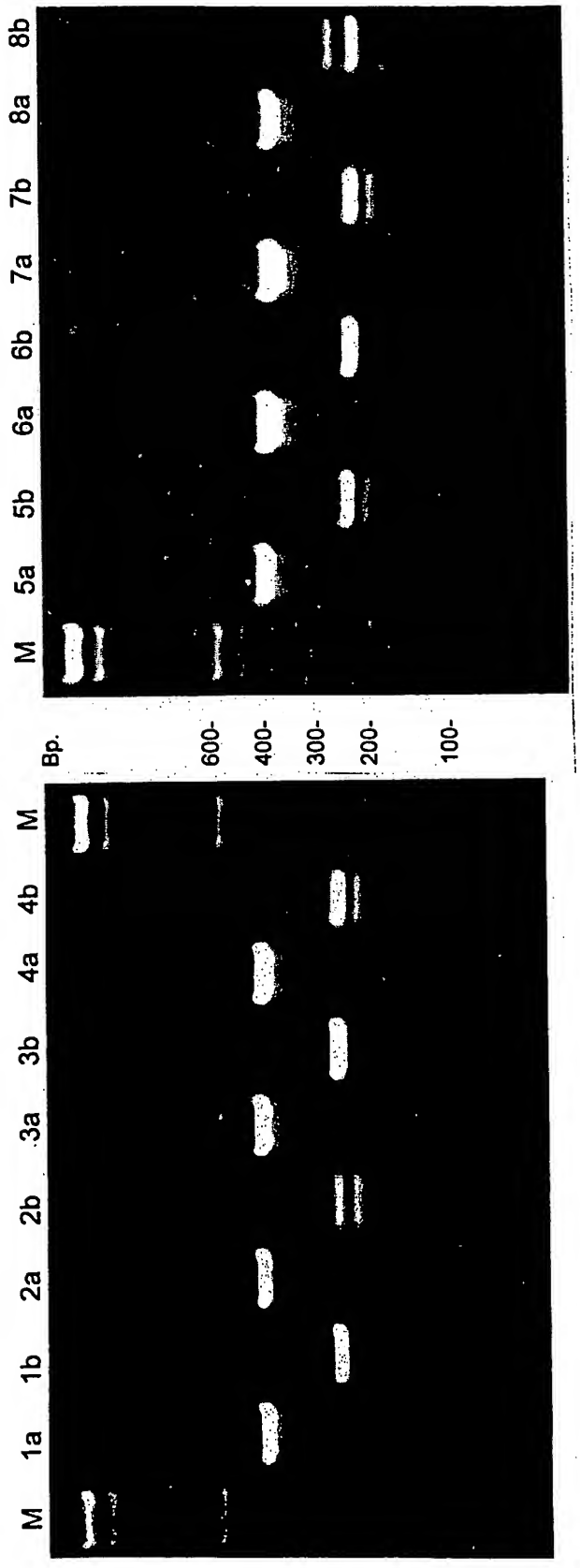

M
5a
5b
6a
6b
7a
7b
8a
8b
Bp.
600-
400-
300-
200-
100-
M
1a
1b
2a
2b
3a
3b
4a
4b
M

Fig. 10A

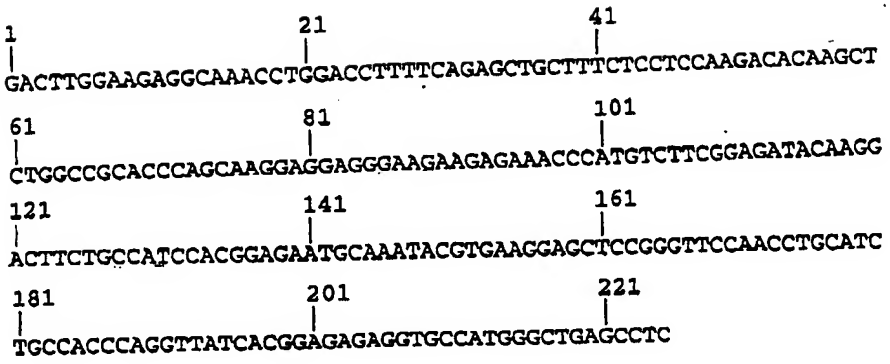
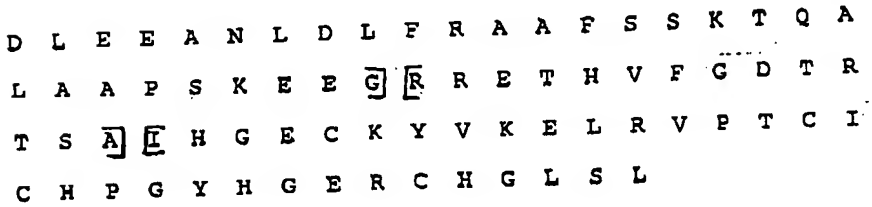


Fig. 10B



INTERNATIONAL SEARCH REPORT

Int. ..ional Application No
PCT/EP 00/05363

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N15/12 C12N15/62 C12N9/00 C07K14/475
C12P21/02 C12Q1/68 A61K38/18 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12P C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, STRAND, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 04688 A (BECHTOLD ROLF ;BIOPHARM GMBH (DE); POHL JENS (DE); UNSICKER KLAUS) 5 February 1998 (1998-02-05) the whole document ---	1-12, 15-28
X	CASPER D ET AL.: "EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture" J. NEUROSCI. RES., vol. 30, no. 2, October 1991 (1991-10), pages 372-381, XP000961063 abstract page 375, right-hand column, line 34-52 --- -/--	1,2,4,5, 7-11, 15-21, 24,25, 27,28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 2000	05/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax (+31–70) 340–3016	van de Kamp, M

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 00/05363

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 06705 A (CHILDRENS MEDICAL CENTER ;CALIFORNIA BIOTECHNOLOGY INC (US)) 30 April 1992 (1992-04-30) claims 1-48 figures 1-5	6-12, 15-21, 24,25, 27,28
X	RAAB G ET AL.: "Heparin-binding EGF-like growth factor" BIOCHIMICA BIOPHYSICA ACTA, vol. 1333, no. 3, 9 December 1997 (1997-12-09), pages F179-F199, XP000961069 paragraphs 2, 3.1 and 3.2, 6.1, 7.1, and paragraph 11	6-12, 15-21, 24,25, 27,28
X	UNSICKER K ET AL: "Growth factor function in the development and maintenace of midbrain dopaminergic neurons: concepts, facts and prospects for TGF-beta" CIBA FOUNDATION SYMPOSIUM, vol. 196, 1996, pages 70-84, XP002050429 abstract tables 1,2 page 75, line 1-13	1,2,4,5, 7-11, 15-21, 24,25, 27,28
A	GOODLAD R A ET AL.: "Epidermal growth factor (EGF)" BAILLIÈRE'S CLINICAL GASTROENTEROLOGY, vol. 10, no. 1, March 1996 (1996-03), pages 33-47, XP000961062 the whole document	1

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 13, 14

Remark (2): Claims 13 and 14 refer to agonists and/or antagonists of the polypeptide(s) without giving a true technical characterisation. Moreover, no specific compounds are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art. 5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/EP 00/05363

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9804688 A	05-02-1998	AU 4202897 A	20-02-1998
		EP 0922101 A	16-06-1999
WO 9206705 A	30-04-1992	AU 663298 B	05-10-1995
		AU 9086991 A	20-05-1992
		CA 2094045 A	17-04-1992
		EP 0559769 A	15-09-1993
		JP 6502538 T	24-03-1994
		US 5811393 A	22-09-1998